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Selective loss of glycogen synthase kinase-3α in birds reveals distinct roles for GSK-3 isozymes in tau phosphorylation

Lina Tsaadon Alon [a,1], Shmuel Pietrokovski [b,1], Shay Barkan [c], Limor Avrahami [a], Oksana Kaidanovich-Beilin [d], James R. Woodgett [d], Anat Barnea [e], Hagit Eldar-Finkelman [a,*]

[a] Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
[b] Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel
[c] Department of Zoology, Tel Aviv University, Tel Aviv, Israel
[d] Department of Medical Biophysics, University of Toronto, Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, Ontario, M5G, 1X5, Canada
[e] Department of Natural and Life Sciences, The Open University of Israel, Raanana, Israel

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ABSTRACT

Mammalian glycogen synthase kinase-3 (GSK-3), a critical regulator in neuronal signaling, cognition, and behavior, exists as two isozymes GSK-3α and GSK-3β. Their distinct biological functions remains largely unknown. Here, we examined the evolutionary significance of each of these isozymes. Surprisingly, we found that unlike other vertebrates that harbor both GSK-3 genes, the GSK-3α gene is missing in birds. GSK-3-mediated tau phosphorylation was significantly lower in adult bird brains than in mouse brains, a phenomenon that was reproduced in GSK-3α knockout mouse brains. Tau phosphorylation was detected in brains from bird embryos suggesting that GSK-3 isozymes play distinct roles in tau phosphorylation during development. Birds are natural GSK-3α knockout organisms and may serve as a novel model to study the distinct functions of GSK-3 isozymes.

1. Introduction

Birds are attractive subjects for research in ecological, neurobiological and behavioral evolution [1,2]. Of particular interest is the neurobiology of birds, which is conspicuously different from that of other vertebrates. Birds display a unique neuroanatomy architecture and are known for their robust neurogenesis and neuronal replacement in comparison with other organisms [3,4]. Birds are also notable for their complex natural behaviors such as vocal communication, learning and social living [1,5]. Recent decoding of the chicken (*Gallus gallus*), domestic turkey (*Meleagris gallopavo*) and zebra finch (*Taeniopygia guttata*) genomes [6–8] should provide information that will reveal biochemical and genomic elements underpinning the unique properties of birds.

Glycogen synthase kinase-3 (GSK-3) is a highly conserved serine/threonine kinase that is a critical regulator of neuronal signaling, cognition and behavior. Abnormal GSK-3 activity promotes cell apoptosis, inhibits neuronal growth and leads to changes in brain morphology and cognitive abilities such as learning and memory and mental and social behavior [9–15]. One important target of GSK-3 is the neuronal microtubule-associated protein tau. Tau is a principle regulator of microtubule stability and an integral player in neuron morphology, outgrowth and plasticity [16–18]. Hyperphosphorylation of tau alters neuronal maturation and plasticity and is a hallmark of the tangle pathology detected in Alzheimer's disease [16,17,19]. GSK-3 phosphorylates tau at several sites [20–22] implicated in Alzheimer's pathogenesis [22–25]. Serine 396 is a unique and a predominant phosphorylation site of GSK-3 [22], and, like many other GSK-3 substrates, its phosphorylation requires prior phosphorylation, in this case at serine 404 [26].

Mammalian GSK-3 exists as two isoforms, α and β, encoded by two different genes [27]. Their distinct biological roles are not fully known. Here we sought to study the functional and evolutionary significance of each GSK-3 isoform. We report that unlike other vertebrates, the GSK-3α gene is missing in birds. Additionally, unlike other substrates tested, lack of GSK-3α resulted in a robust reduction in tau phosphorylation in the adult bird brain.

2. Materials and methods

2.1. Materials

Monoclonal antibody that recognizes GSK-3α and β was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA),

* Corresponding author. Fax: +972 3 640 8749.
E-mail address: heldar@post.tau.ac.il (H. Eldar-Finkelman).
[1] These authors contributed equally to this work.

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and anti-phosph-GSK-3 (Tyr 216/279) was obtained from Millipore (Billerica, MA, USA). Anti-β-catenin, anti-phospho-CREB (Ser 129/133) and anti-tau were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti phospho-tau antibodies (Ser 202, Ser 396, Ser 404) were from Cell Signaling Technologies and Millipore.

2.2. Bioinformatic analysis

Sequence searches of NCBI databases were performed using the BLAST programs. Protein, genomic and transcribed (EST) sequence databases were queried with known GSK-3 sequences. Potential GSK-3 protein sequences were further specifically examined to verify their identity. Sequences of GSK-3 proteins were aligned using BLASTp, meme [28] and GLAM-2 [29] programs and the corresponding coding regions were edited with the Se-Al sequence alignment editor (Andrew Rambaut, http://tree.bio.ed.ac.uk/software/seal/). A phylogenetic tree was calculated with the PHYML program, version 2.4.4, with four substitution rate categories, the HKY nucleotide substitution model and other default parameters to estimate the Ts/Tv ratios, gamma shape parameters and invariant proportions. These parameters were then used with 100 bootstrap replicates. Corresponding trees were found with different combinations of GSK-3 sequences and with the RaxML program [30] (data not shown).

2.3. Animals

Zebra finches were obtained from a breeding colony in Tel Aviv University, and house sparrows were collected under the Israel Nature and National Parks Protection Authority permit. Chicken embryos were used at day 18 (three days prior to hatching). GSK-3α KO mice and their wild-type littermates were generated and maintained as previously described [10,31]. Animals were housed in the Toronto Center for Phenogenomics (TCP) in Toronto, Canada. Hippocampal samples for western blot analysis were isolated from 4 month old GSK-3α KO ($n = 4$) and wild-type (WT) mice ($n = 4$). Animal use was approved by the Tel Aviv University Institutional Animal Care and Use Committee or TCP as appropriate.

2.4. Tissues

Brains, livers and hearts were removed from birds (adult or embryos as indicated) or mice and frozen in liquid nitrogen. Frozen tissues were homogenized with buffer H (10 to 250 mM β-glycerophosphate, 10% glycerol, 1 to 7.5 mM EGTA, 1 to 5 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM orthovanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 25 μg/ml aprotinin, 5 μg/ml pepstatin, and 0.5% Triton X100). Equal amount of proteins (60 μg) were subjected to gel electrophoresis followed by immunoblot analysis using indicated antibodies.

2.5. Statistics

Data were analyzed with Origin Professional 6.0 software using the Student's *t*-test. Data were considered significant at $p < 0.05$.

3. Results

3.1. GSK-3α gene is missing from bird genomes

By comparing GSK-3 sequences from diverse organisms (Fig. 1A and S1), we found that the α and β isoforms split from a common precursor approximately at the time of emergence of vertebrates. Surprisingly, although both GSK-3α and β genes are present and highly conserved in fish, amphibians, reptiles and mammals, the GSK-3α gene was missing from the genomes of chicken, domestic turkey and zebra finch, the three bird species with available draft genomes and extensive transcriptomic data [6–8]. The absence of GSK-3α from these genomes and transcriptomes of three diverse bird species suggests this is a common loss in the avian species. The presence of GSK-3α in various fish, amphibians and lizards clearly indicates that the GSK-3α gene was lost after the split of ancestral birds from reptilians. Sequence identities between GSK-3β from human and chicken and between human and zebra finch are 96% and 94%, respectively.

To verify our findings experimentally, protein expression patterns of GSK-3α and β were examined by western blot analysis in mouse and three different bird species: domestic chicken, house sparrow (*Passer domesticus*) and zebra finch. Two different antibodies were used; both recognize highly conserved regions within GSK-3α and β. One monoclonal antibody recognizes both GSK-3s and the other recognizes a phospho-tyrosine (Tyr 279 and Tyr 216 of GSK-3α and β, respectively). The two bands corresponding to GSK-3α and β were clearly observed in mouse brains (Fig. 1B), but GSK-3α was not detected in samples from the brains of any of the birds (Fig. 1B) or in other bird tissues such as heart and liver (Fig. 1C).

3.2. Lack of GSK-3a results in reduced tau phosphorylation in the adult bird brain

We next examined if lack of GSK-3α in birds affects tau phosphorylation. Brain homogenates of birds (house sparrow and zebra finch) were subjected to western blot analysis using various anti-phospho-tau antibodies. A significant reduction in tau phosphorylation at the known GSK-3 phosphorylation site, Ser 396 [22], was detected in the samples from bird's brain as compared with those from mouse brain (Fig. 2A, tau isoforms migrate as 52–72 kDa bands). This was not due to loss of tau, as 'total' tau was readily detected in bird brains (Fig. 2A). The data demonstrating that the anti-phospho-tau antibody recognizes bird tau is discussed in the following section. As shown in Fig. 2A, the bird tau was phosphorylated at the 'priming site' Ser 404 [26], and at Ser 202, a site phosphorylated by multiple kinases including GSK-3 [20] (Fig. 2A). Quantification of the phosphorylated bands revealed no significant differences in the ratios of phosphorylated tau (Ser 404, Ser 202) to total tau in birds or mice (Fig. 2A). These data demonstrated that tau can be phosphorylated at additional sites and validated the significance in the observed reduction of tau phosphorylation at Ser 396 in bird brain.

β-Catenin is a key effector in the Wnt signaling pathway and a known substrate of GSK-3 [32]. Phosphorylation of β-catenin by GSK-3 enhances its proteosomal degradation [32]. Hence, lack of GSK-3α could lead to elevation in β-catenin levels. However, β-catenin levels in bird brains were comparable to those observed in the mouse brain (Fig. 2B). Similarly, no significant changes were observed in the phosphorylation of another GSK-3 target-CREB (cAMP response element binding protein) [33]. Quantification of phosphorylated CREB revealed no significant differences in the ratios of phospho-CREB to total CREB in birds vs. mice.

Finally, experiments were conducted in brains of GSK-3α knockout (KO) mice [31]. As shown in Fig. 2C, phosphorylation of tau at Ser 396 was significantly lower in brains from GSK-3α KO mice as compared those of WT mice. No significant differences were observed in β-catenin levels or the ratios of phospho-CREB to total CREB in GSK-3α KO vs. WT brain samples (Fig. 2C). It appears that GSK-3 isoforms are functionally redundant in regulation of β-catenin (as also shown in [34]) and CREB phosphorylation. In

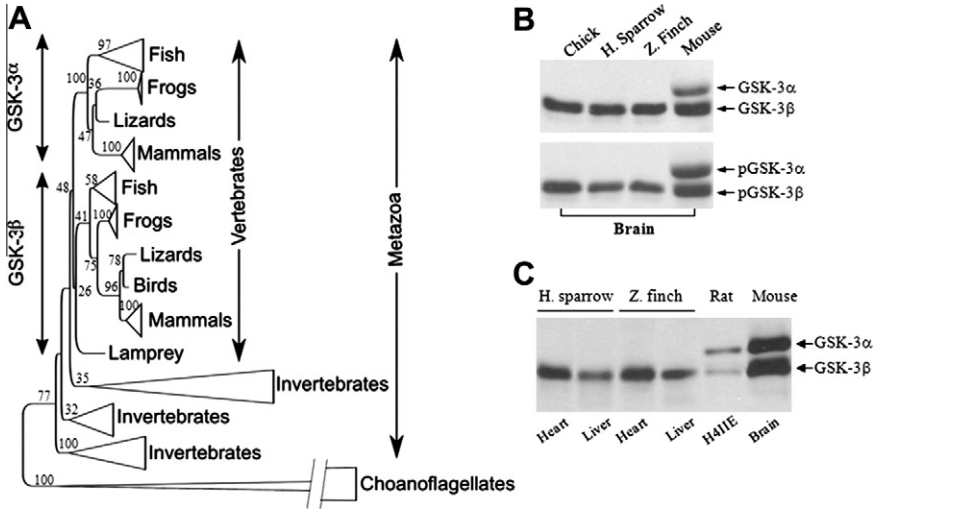

Fig. 1. GSK-3α is missing in birds. (A) Schematic representation of a phylogenetic tree calculated from multiple sequence alignment of diverse GSK-3 sequences (see Fig. S1 for complete data). Triangles cluster several species of the noted groups. Alpha and Beta labeled arrows show groups where GSK-3α and GSK-3β sequences, respectively, were found. Note that a GSK-3 gene from a lamprey (*Petromyzon marinus*), a basal jawless vertebrate, was found within the GSK-3β group and that GSK-3 sequences from non-vertebrate basal chordates (*Amphioxus*, a cephalochordate, and *Saccoglosus*, a hemichordate) were placed in groups outside the α and β GSK-3 sequences (Fig. S1). The unicellular choanoflagellates (the ancestors of sponges) were used as the out group to root the tree. No GSK-3α genes were found in birds. Bootstrap values are shown. (B) Expression of GSK-3 proteins in brains of chicken embryo (day 18), house sparrow, zebra finch and mouse. Brain tissue extracts (60 μg) were subjected to western blot analysis using anti-GSK-3 (upper panel) or anti-phospho-GSK-3 (Tyr 216/279) (lower panel) antibodies. Shown are representative gels from three independent experiments.

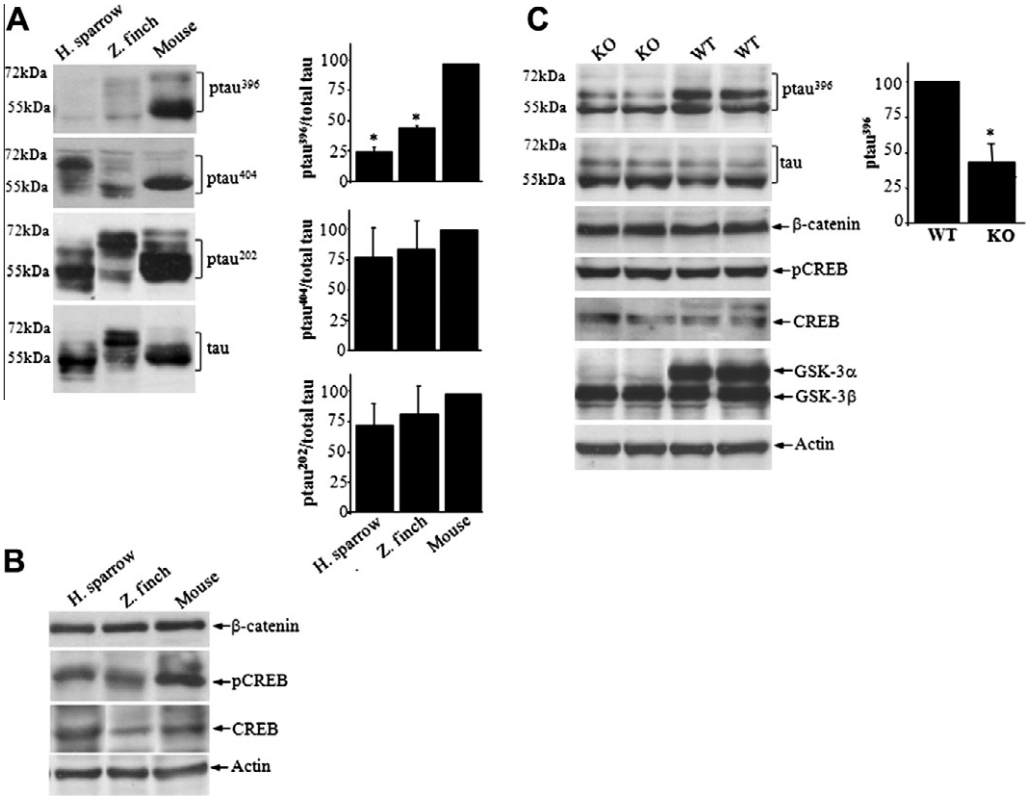

Fig. 2. Lack of GSK-3α results in reduced tau phosphorylation. (A) Brain tissue extracts (60 μg) prepared from house sparrow, zebra finch and mouse were subjected to gel electrophoresis and western blot analysis using the indicated anti-phospho-tau (Ser 396, Ser 404 and Ser 202) antibodies. Densitometry analysis of the phosphorylated fraction of tau (phospho-tau/total tau) is shown in the right panel and is fold of the phosphorylated fraction obtained for mouse tau that was designated to 100%. Results are mean ± S.E.M. ($n = 3$). Tau isoforms migrate as proteins ranging from 52 to 72 kDa. (B) The same as A except that anti-β-catenin, anti-phospho-CREB (Ser129/133), and anti-CREB antibodies were used. Expression of actin is shown to confirm equal loading. (C) Hippocampus tissue extracts (60 μg) prepared from GSK-3α KO and WT were subjected to western blot analysis using the same antibodies described in (A). Densitometry analysis of phosphorylated tau is shown in the right panel as a mean ± S.E.M. ($n = 4$). Gels are representative of three individuals of each species of bird and four KO and four WT mice.

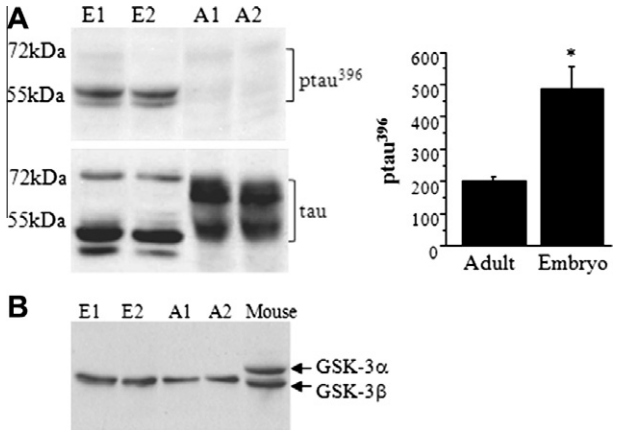

Fig. 3. Phosphorylation of tau in bird embryos. (A) Phosphorylation of tau in embryonic brain. Tissue extracts prepared from brains of zebra finch embryos (day 9–10 days) or adults were subjected to western blot analysis using anti-phospho-tau and anti-tau antibodies. Densitometry analysis of phospho-tau (Ser 396) is shown in the right panel as mean ± S.E.M. ($n = 3$). (B) The same experiment as described A except that anti-GSK-3 antibody was used and a mouse brain sample was added as a reference. Expression of GSK-3α and GSK-3β is shown.

contrast, GSK-3α-mediated tau phosphorylation is not augmented by GSK-3β, suggesting that GSK-3 isoforms have distinct properties with respect to tau phosphorylation.

3.3. Role for GSK-3β in phosphorylating embryonic tau

Our results raised the possibility that GSK-3β is unable to phosphorylate tau (Fig. 2A). On the other hand, phosphorylation of tau could be dependent on cellular context. It is known that expression and phosphorylation of tau is developmentally regulated [35–37] and this prompted us to evaluate tau phosphorylation in the bird embryos. Brain homogenates prepared from zebra finch embryos (day 9–10) were subjected to gel electrophoresis and blots were probed for phosphorylated tau. Different tau isoforms were detected in embryos than in adult brain (Fig. 3A). Phosphorylation at Ser 396 was very weak in adult brains, whereas it was readily detected in the embryonic samples (Fig. 3A). Notably, these results confirmed the ability of the anti-phospho-tau antibody to recognize bird tau. In addition, we show that embryonic tau phosphorylated at Ser 396 was comparable with that of mouse further validating the ability of this antibody to recognize bird's tau (Fig. S2)). Since GSK-3β is the isoform expressed in the embryo (Fig. 3B), it is suggested that GSK-3β is responsible for the phosphorylation of the embryonic tau isoforms, although, we cannot ignore the possibility the other protein kinases such as mitogen activated protein kinase (MAPK) and CDK-5 may contribute to tau phosphorylation at this site as well [20].

4. Discussion

Gene duplication is considered a fundamental mechanism for functional innovation during evolution [38]. The emergence of two GSK-3 isozymes and their retention during vertebrate evolution suggests an important diversity in their biological functions. Although we do not know how the GSK-3α gene was deleted from the bird lineage or why, the lack of GSK-3α likely influenced the biology of the avian species. It is possible that high doses of GSK-3 activity (resulting from expression of two genes) had deleterious effects in birds, and that this was remedied by deletion of the GSK-3α gene from the ancestral bird genome. Studies in mammalian models indicate that lack of GSK-3α or inhibition of GSK-3 activity alters neuronal architecture and cognitive activities [10–12,14]. For example, abnormalities in brain structure were observed in GSK-3α knockout mice [10], and inhibition of GSK-3 or deletion of one allele of GSK-3β mimicked the activity of the mood stabilizer lithium and improved depressive-like behavior [14,39]. Interestingly, hyperactivity of GSK-3 in mouse brain results in socialization deficits [12]. It is thus tempting to speculate that lack of GSK-3α contributed to the unique brain organization and anatomy of birds and was responsible, at least in part, for their complex cognitive abilities including navigation, learning and memory and social behavior [1,2]. The dramatic reduction in tau phosphorylation at the known GSK-3 phosphorylation site observed in bird brains (a phenomenon that was reproduced in brains of GSK-3α KO mice), as well as the demonstrated role of reduced tau phosphorylation in neuronal activity and synaptic plasticity [17,40,41], suggests a possible link between lack of GSK-3α, tau, and avian brain function.

Little is known about tau protein in birds. One study characterized tau from chicken and confirmed its ability to promote microtubule assembly [37]. It also showed that tau expression was developmentally regulated, and that its phosphorylation was more extensive in the embryo than after hatching [37]. We report here a similar observation in which tau phosphorylation at Ser 396 was significantly higher in zebra finch embryos than in adult brains. This site is predominantly phosphorylated by GSK-3 [22,26] (although we should note that other protein kinases such as MAPK and CDK-5 were shown to phosphorylate this site [20]), indicating that GSK-3β can phosphorylate embryonic tau. This raises the supposition that GSK-3α and GSK-3β have distinct roles in phosphorylating tau in adult and embryonic brain in non-bird vertebrates. Future studies should investigate this question. Finally, inhibition of GSK-3 and reduced tau phosphorylation is thought to benefit patients with Alzheimer's disease [42–44]. Our data support the hypothesis that specific inhibition of GSK-3α may be useful for therapeutic intervention in these diseases. In summary, birds might be regarded as natural GSK-3α knockout organisms and will serve as a novel model to further our understanding of the distinct functions of GSK-3 isoforms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.03.025.

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